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Interaction of Group B Streptococcal Opacity Variants with the Host Defense System

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Group B streptococci (GBS) demonstrate high-frequency phase variation of colony opacity. Colony opacity is a function of chain length, with opaque colonies consisting of GBS that form longer chains. Because opaque variants do not grow on standard streptococcal media, the role of opacity variation in GBS infection has not been studied. We have isolated stable variants from type III GBS that are either transparent (variants 1.2 and 1.3) or opaque (variants 1.1 and 1.5). In this study, we evaluated the interactions of these variants with different components of the host immune system both in vitro and in vivo. Opaque GBS were less immunogenic than transparent GBS. Opaque GBS were more susceptible to killing by polymorphonuclear neutrophils (PMNs) and could induce a chemiluminescent response of PMNs in the absence of antibody (Ab) or complement. Transparent GBS did not induce neutrophil chemiluminescence in the absence of Ab and complement. However, in the presence of Ab and complement, transparent GBS induced a stronger chemiluminescent response than did opaque GBS. Scanning electron micrographs of PMNs and GBS demonstrated differences in the attachment and engulfment of the different variants by the PMNs as well as different effects of the GBS on the PMNs themselves. Interactions with complement were affected by GBS opacity as well, with opaque variant 1.1 initiating complement activation in the absence of any Ab. The virulence of the GBS opacity variants was studied in vivo by inoculation of graded numbers of GBS into newborn mice. Transparent variants 1.2 and 1.3 were most virulent, with variant 1.1 intermediate and variant 1.5 minimally virulent. However, in mixed infections, variant 1.5 greatly enhanced the virulence of small numbers of transparent GBS. These results indicate that the opacity status of GBS can influence the interaction between the GBS and the host immune system.

Group B streptococci (GBS) may cause different clinical syndromes. They may asymptomatically colonize the female genital tract, where they may or may not provoke an antibody (Ab) response. Neonates exposed to GBS during birth are at risk of developing disease if the mother has not produced an Ab response to the colonizing GBS. Neonatal disease may be either a rapidly progressive sepsis appearing within the first few days of birth or a more indolent illness in which meningitis develops at 1 to 4 weeks of age. Although bacterial virulence factors have been defined (10), differences in clinical presentation of GBS infection have traditionally been attributed to host factors (1, 2, 4).

We have recently described high-frequency phase variation of GBS (5). Variation in chain length was manifested as changes in the opacity of bacterial colonies. Stable opaque and transparent variants of type III GBS were isolated, and their physical characteristics were studied. Opaque bacteria differ from transparent GBS in several important ways, including antigenicity, capsular structure, and cell surface protein profile. Because opaque GBS grow poorly, if at all, in Todd-Hewitt medium, the standard medium used to grow GBS for experimental studies, the role of opacity variation in the life cycle of GBS and the pathogenesis of GBS infection remains to established.

In this study, we have evaluated the interactions between the different opacity variants and the host defenses. Interactions with Ab, complement, and polymorphonuclear neutrophils (PMNs) were studied in vitro. In vivo analyses of immunogenicity and virulence were performed. The results show that the opacity variants have distinctly different interactions with the immune and inflammatory systems. The ability of the GBS to switch opacity type may allow the GBS to escape from local host defenses and thus may play a role in the pathogenesis of GBS infection.

MATERIALS AND METHODS

Bacteria, Ab, and complement. Opacity variants 1.1, 1.2, 1.3, and 1.5 were derived by serial passage from a clinical isolate of type III GBS and were maintained on GC typing medium (5). Characteristics of these variants are summarized in Table 1. Monoclonal Ab (MAb) S9 is immunoglobulin M directed against the sialic acid-containing epitope on the type III GBS capsule (6). Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin was purchased from Zymed Laboratories (South San Francisco, Calif.). Complement used in opsonophagocytosis assays was lyophilized rabbit complement (Pel-Freez, Brown Deer, Wis.) that was reconstituted just prior to use. Complement used in assays of complement consumption was a human complement standard obtained from Kallestad Laboratories (Austin, Tex.).

Immunizations and immunoassays. To test the immunogenicity of GBS variants, groups of five BALB/c mice were immunized with live GBS in saline, given intraperitoneally. Bacteria were suspended to an optical density of 0.9, and 0.1 ml was injected per mouse. Mice were injected on days 1, 20, and 27 and bled on days 0 (prebleed), 20 (primary bleed), 27 (secondary), and 34 (tertiary).

Abs were studied by enzyme-linked immunosorbent assay

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TABLE 1. Characteristics of GBS opacity variants[a]

Variant	Opacity[b]	Chain length[c]	CFU (10^6)/ml[d]	Capsule[e]	Group antigen[f]	Variation[g]
1.1	+	4–6	104	+/−	+	+
1.2	−	2	480	+	+	+
1.3	−	2	550	+	+	+
1.5	++	>10	58	+	−	−

[a] Data summarized from reference 5.
[b] Colony opacity as observed under a dissecting microscope with oblique light. +, opaque; −, nonopaque or transparent.
[c] Number of attached bacteria per chain as observed by transmission electron microscopy.
[d] GBS were suspended at an optical density at 600 nm of 0.9 and then serially diluted and plated, and colonies were counted.
[e] Presence of capsule as determined by Abs, buoyant density, and electrophoretic mobility.
[f] Presence of group antigen as determined by Abs.
[g] Ability to change opacity state.

(ELISA) and Western blotting (immunoblotting). The ELISA to measure Ab to intact GBS has been described elsewhere (6). Briefly, intact GBS were adhered to the wells of 96-well microtiter plates. Dilutions of the sera to be tested were added to the wells and incubated for 2 to 6 h. The wells were washed, and alkaline phosphatase-conjugated anti-mouse immunoglobulin was added. Following an overnight incubation, the wells were washed, and the chromogenic substrate *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) in 10% diethanolamine (pH 9.8) was added. The A_{405} was determined at 20 to 60 min. In one experiment, GBS were treated with proteinase K (0.5 mg/ml; Sigma) for 60 min at 55°C prior to use in ELISA. Western blots were performed with bacteria lysed with mutanolysin and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (5). The gels were blotted onto nitrocellulose filters (BA85; Schleicher & Schuell, Inc., Keene, N.H.) and incubated with the serum to be tested. Following washing, the nitrocellulose was incubated with ^{125}I-protein A (Dupont, NEN Research Products, Boston, Mass.) and then washed again, and autoradiography was performed.

PMN assays. PMNs were purified on a stepwise density gradient of Ficoll-Paque (densities, 1,119 and 1,077 gm/liter; Sigma). Fresh heparinized blood was diluted 1:2 in Hanks balanced salt solution and layered on top of the gradient. Cells were spun at 700 × *g* for 30 min. The PMNs were found at the interface of the 1,119- and 1,077-g/liter layers. Cells were removed, washed, and resuspended in Hanks balanced salt solution with 0.1% gelatin. Purity of PMNs was assessed by microscopic examination.

Survival of GBS in the presence of PMNs was examined by mixing GBS (A_{600} of 0.9; diluted 1:50 in phosphate-buffered saline [PBS]; 50 μl added) and PMNs (10^6) in a total volume of 0.5 ml. Bacteria and PMNs were tumbled at 37°C for 2 h. Serial dilutions were then plated on GC typing medium, and the number of colonies was determined the next day. Percent survival was determined by comparing the results obtained in the presence and absence of PMNs.

Luminol-enhanced chemiluminescence was performed in the presence or absence of Ab and complement. The follow-

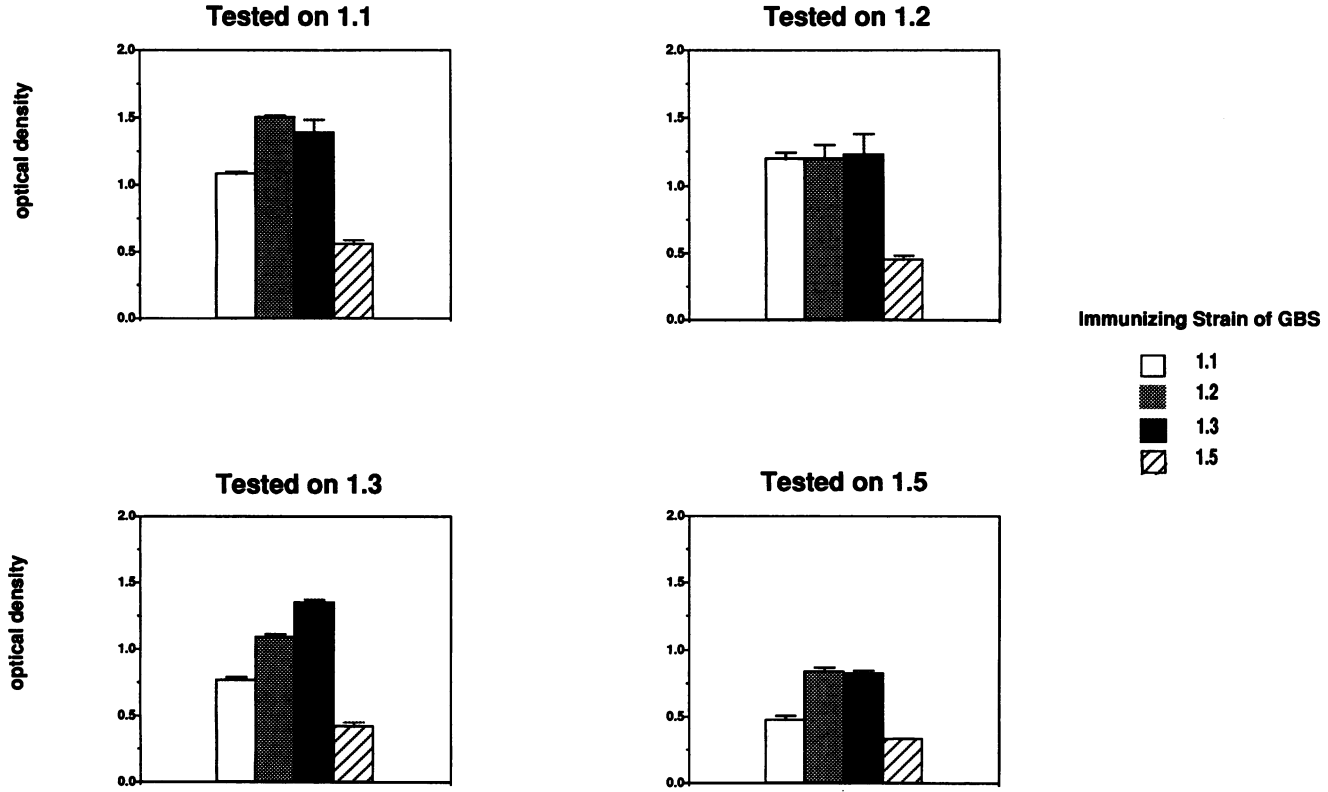

FIG. 1. Binding of mouse antisera to GBS variants. Mice were immunized three times with live GBS variants 1.1, 1.2, 1.3, and 1.5. Pooled sera from each group were tested by ELISA against intact bacteria of each variant. Serum Ab titers were measured over a range of dilutions. Shown are the A_{405}s from the 1:5,000 dilution. Data are means and standard errors of the means of triplicate values.

ing were mixed in the dark to a final volume of 1.0 ml of Hanks balanced salt solution–0.1% gelatin: PMN (10^5), MAb S9 (250 ng), rabbit complement (final dilution, 1:160), and luminol (10^{-5} M). The mixture was shaken and counted on a liquid scintillation counter (Beckman model LS9000) with the window set at 0 to 397 to determine the background level of counts. GBS were added (A_{600} of 0.9; diluted 1:50 in PBS; 50 μl added), and the samples were run continually on the scintillation counter for 120 min.

SEM. PMNs, GBS, Ab, and complement were mixed in the same proportions as for chemiluminescence and tumbled together for 1 h at 37°C. The PMNs and GBS were sedimented onto scanning electron microscopy (SEM) grids at 1 × *g* for 2 h. Samples were fixed in 3% glutaraldehyde and then 2% osmium tetroxide in 0.1% sodium cacodylate buffer. The fixed samples were coated with 10-nm gold-palladium and were examined in a model 35 CF scanning electron microscope (JEOL, Peabody, Mass.). To score the characteristics of the PMNs, photographs were made of 40 consecutive cells as the field was scanned. The photographs were separately evaluated by two blinded scorers. The PMNs were scored for the number of adherent GBS per cell, ruffling (0, smooth; 1, occasional folds; 2, mixed smooth areas and folded areas; 3, continuous folds), and number of pseudopods (defined as membrane-bound tubular projections <0.45 μm in diameter and with a length at least three times the diameter) per cell.

Complement activation. Complement activation by GBS was measured by using two different assays that measure the depletion of total hemolytic complement from a standard serum. GBS (A_{600} of 0.9) were mixed with MAb S9 (15 μg/ml) or PBS for 1 h at 37°C and then washed. Standard complement serum was added to a final dilution of 1:6, and the samples were incubated for 45 min at 37°C. The GBS were spun out, and the supernatants were then tested for total hemolytic complement by two different assays. In the first, serial dilutions of the serum were placed in the wells of a radial diffusion plate containing sensitized sheep erythrocytes (SRBC) in agarose (plates were purchased from Kallestad Laboratories). The zone of SRBC lysis was measured after an overnight incubation at 37°C. Two measurements were performed at right angles on each of duplicate samples. The second assay was performed on sensitized SRBC in suspension. SRBC were sensitized with rabbit anti-SRBC sera (Diamedix Laboratories, Miami, Fla.) at a 1:500 dilution. Serial dilutions of the samples to be tested were added to a 0.3% suspension of sensitized SRBC and incubated with tumbling at 37°C for 40 min. Intact SRBC were removed by centrifugation, and the A_{514} of the supernatant was determined.

In vivo infections. Outbred RML mice were infected by intraperitoneal injection at 0 to 24 h of age. GBS were suspended to an A_{600} of 0.9, and each mouse was injected with 10 μl of the original suspension or serial dilutions. Mice were monitored until time of death. Experimental groups consisted of six neonatal mice caged with a single lactating mother except in the mixed-infection experiment, for which litters of 10 mice were used. To determine tissue distribution and opacity type of GBS following infection, mice were sacrificed 18 to 48 h postinfection, and tissue lysates were serially diluted and cultured. All experimental protocols were approved by the Rocky Mountain Laboratories Animal Care and Use Committee.

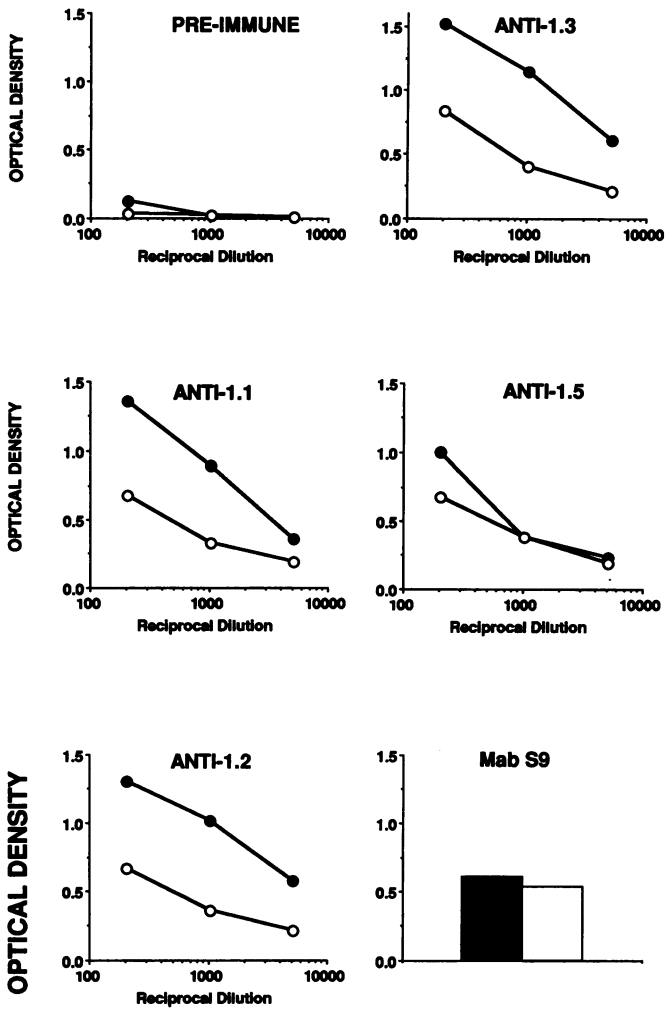

FIG. 2. Binding of mouse antisera to intact and proteinase K-treated GBS. GBS variant 1.2 was either left intact (●) or treated with proteinase K (○). Pooled sera from groups of mice immunized with each of the variants were tested for binding to the two preparations of GBS by ELISA. MAb S9, which binds to the type III carbohydrate, was used as an internal control. Filled symbols, binding to intact GBS; open symbols, binding to proteinase K-treated GBS.

RESULTS

Immunogenicity. Mice were immunized with live GBS variants 1.1, 1.2, 1.3, and 1.5, five mice per group. All mice received three immunizations. Serum Ab titers were measured by ELISA on whole GBS, using each of the variants. Peak Ab titers were obtained in each group after the secondary immunization; there was no further increase in Ab following the third immunization. Figure 1 shows the binding of pooled sera from tertiary bleeds of each group of mice tested on the different GBS variants. The sera raised against variant 1.5 demonstrated the least binding against every strain of GBS, including the immunizing strain. Although only a single dilution is shown in Fig. 1, this observation was true over the entire range of dilutions tested (1:200 to 1:5,000). Compared with the titer obtained from immunization with the transparent strains of GBS (variants 1.2 and 1.3), the variant 1.5-immune mice produced Ab with at least a 25-fold decrease in titer (not shown). Mice immunized with

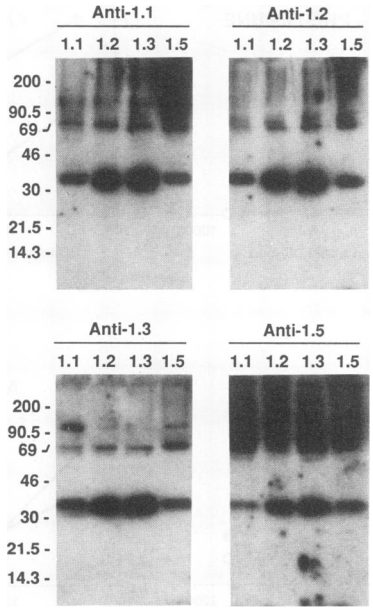

FIG. 3. Western blots performed with mouse antisera to GBS variants. Lysates of each GBS variant were subjected to SDS-PAGE and then blotted against pooled sera from mice immunized with each of the different opacity variants. Ab binding was detected with radiolabeled protein A. Positions of size markers (in kilodaltons) are indicated on the left.

variant 1.1 also produced less Ab than did those immunized with the transparent strains of GBS, although the decrease was not as marked (at most a 10-fold decrease in titer). Surprisingly, the greatest difference between the sera against the opaque and transparent GBS was seen when the Abs were tested on the opaque GBS. Similar results were seen when sera from individual mice, rather than serum pools, were tested. It is unlikely that these results are due to a difference in the virulence of the live GBS causing an altered exposure to the GBS, since a tertiary immunization failed to increase the titer of Ab to any of the GBS variants. Thus, the opaque GBS are less immunogenic than the transparent GBS.

We have determined the specificity of the Ab response to the different variants. Surprisingly, very little Ab was detected with use of ELISA to purified type III or group B carbohydrate antigens (7). When antibody binding to proteinase K-treated bacteria was compared with that seen in intact bacteria, it was apparent that the majority of Ab elicited by variants 1.1, 1.2, and 1.3 was directed against protein antigens, while virtually none of the anti-variant 1.5 Ab was antiprotein (Fig. 2). These data indicate that the reason for the diminished Ab response to variant 1.5 is due to the inability of this variant to elicit antiprotein Abs.

We have also compared the resulting sera for reactivity with different components of the bacteria by using the Western blot technique (Fig. 3). Again, the pooled sera from each group were tested against each GBS variant. The only significant difference seen was in the mice immunized with GBS variant 1.5, which reacted with a high-molecular-weight heterogeneous material that is most likely a bacterial polysaccharide, since it is not sensitive to proteinase K digestion (data not shown). Other antisera also recognize this material when tested on variant 1.5. The predominant protein recognized by all sera ran at 35 kDa. This protein

TABLE 2. Survival of variants in the presence of PMNs[a]

Variant	% Survival
1.1	16.8
1.2	73.1
1.3	84.3
1.5	0.8

[a] GBS and PMNs were incubated together for 2 h at 37°C. Percent survival was determined by comparing the number of colonies seen following incubation in either the presence or absence of PMNs.

appears to be present in greater quantity in the transparent GBS. Other immunogenic proteins are seen at 69 and 120 kDa. We have previously identified a 46-kDa protein that is surface exposed and overexpressed in variant 1.1 (5). This protein does not appear to be immunogenic, since the anti-variant 1.1 antiserum fails to react with this material.

Interactions with PMNs. Interactions of GBS with PMNs play an important role in the virulence of GBS (4, 9). We have examined the interactions of the GBS opacity variants with PMNs in several different ways. We tested the ability of PMNs to kill the GBS variants in the absence of antibody or complement (Table 2). The opaque variants were much more sensitive to nonopsonic killing by PMNs, with variant 1.5 being the most sensitive.

To determine whether the increased sensitivity of the GBS variants to PMN killing was due to activation of PMN cytotoxic activity by the opaque variants or just a function of increased fragility of these bacteria, we measured the ability of the GBS variants to induce PMN chemiluminescence.

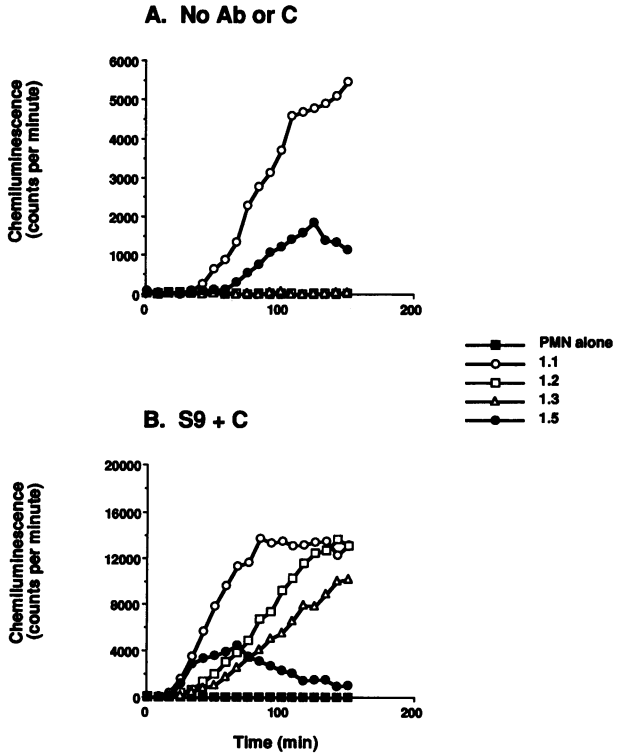

FIG. 4. PMN chemiluminescence induced by GBS opacity variants. PMNs were incubated with GBS opacity variants in the absence (A) or presence (B) of MAb S9 (250 ng/ml) and complement (C; rabbit serum; 1:160). Luminol-enhanced chemiluminescence was measured in a scintillation counter at room temperature over a 3-h period.

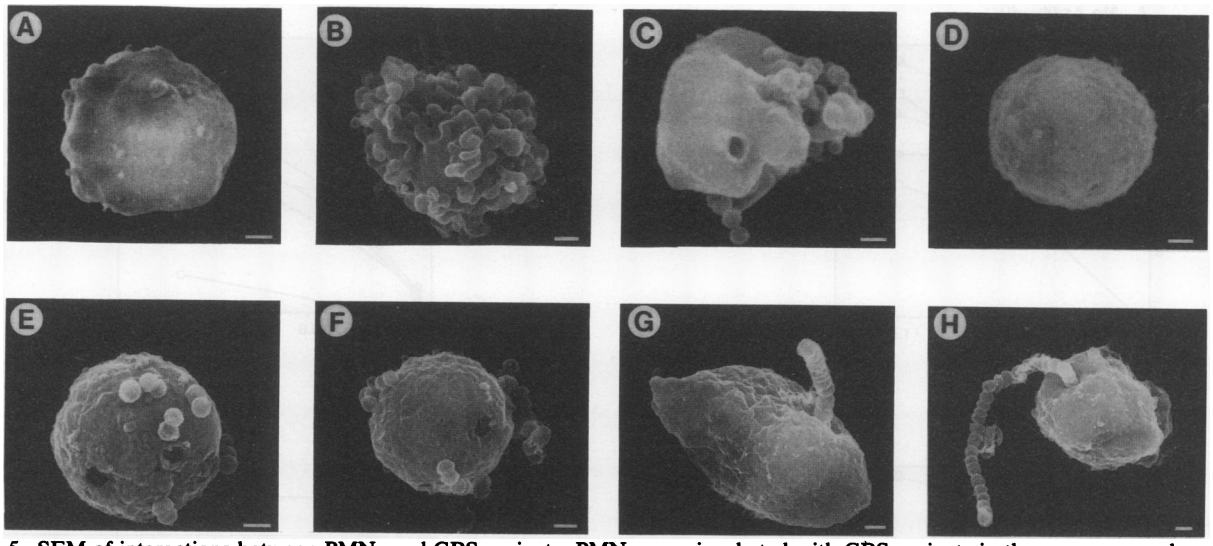

FIG. 5. SEM of interactions between PMNs and GBS variants. PMNs were incubated with GBS variants in the presence or absence of MAb S9 (250 ng/ml) and complement (rabbit serum; 1:160). Bacteria and PMNs were sedimented onto SEM grids and fixed. Each figure shows representative PMNs from the different experimental groups. Bars, 1 μm. (A) No GBS, Ab, or complement; (B) variant 1.1, no Ab or complement; (C) variant 1.1, Ab and complement present; (D) variant 1.2, no Ab or complement; (E and F) variant 1.2, Ab and complement present; (G and H) variant 1.5, Ab and complement present.

Luminol-enhanced chemiluminescence was studied in both the presence and absence of Ab and complement (Fig. 4). Opaque variants 1.1 and 1.5 were able to induce low levels of PMN chemiluminescence in the absence of Ab and complement, whereas the transparent GBS gave none. However, when the anticapsular MAb S9 and complement were added, the transparent variants 1.2 and 1.3 showed excellent responses, whereas the opaque strains produced a lower level of chemiluminescence. The lower chemiluminescent response induced by opaque GBS in the presence of Ab and complement was not due to an inability of MAb S9 to bind to these strains of GBS, since we have shown by ELISA that MAb S9 binds equally to intact bacteria of each variant (5). The addition of the PMN-activating cytokine granulocyte-macrophage colony-stimulating factor to the mixture does not alter these results, although the net response invoked in each case is somewhat larger in the presence of this cytokine (data not shown).

To analyze the interactions between the GBS variants and PMNs in another manner, we have performed SEM. GBS variants and PMNs were incubated together in either the presence or absence of S9 and complement and then sedimented onto SEM grids. In Fig. 5 we show representative micrographs, and in Table 3 we summarize the results of a blinded analysis of 40 consecutive PMNs. Several conclusions may be drawn from the SEM analyses. (i) Opaque variant 1.1 induces a profound perturbation of the surface of the PMNs in the absence of Ab and complement. Surprisingly, when Ab and complement are present, this is not seen. Although the micrographs do not indicate that attachment of GBS to PMN was necessary for this effect, we were unable to duplicate this effect with the broth from a culture of variant 1.1, suggesting that it was not due to a secreted factor. (ii) There was no attachment of GBS to PMNs in the absence of Ab and complement. In the presence of Ab and complement, the attachment of different GBS variants to PMNs varied. The transparent GBS variants 1.2 and 1.3 were attached to the PMNs at multiple points along the cell surface, with each individual bacterium adhering to the surface of the PMNs (Fig. 5E and F). In contrast, the opaque variant 1.5 had only a single point of attachment within the very long chains of these GBS (Fig. 5G and H). Variant 1.1 was intermediate. In addition, there were a greater number of the transparent GBS associated with each PMN. (iii) Morphological criteria of a PMN response (ruffling, pseudopod formation) did not always correlate well with the level of activation as measured by luminol-enhanced chemiluminescence.

Complement activation. Two different assays for the measurement of total hemolytic complement were used to measure the consumption of complement initiated by the opacity variants. Depletion of complement from a standard serum indicated that the complement cascade was activated. These assays were performed both in the presence of MAb S9 and in its absence. GBS were first incubated in the presence of MAb or PBS, washed, and then incubated with the standard serum. After the GBS were spun out, the serum was tested for the presence of complement. The first assay used radial diffusion in a plate containing sensitized SRBC to measure the concentration of hemolytic complement (Fig. 6). In the presence of MAb S9, complement was consumed equally by all four strains, with the majority of complement activity

TABLE 3. SEM analysis of GBS-PMN interactions[a]

Variant	MAb S9 + rabbit complement	No. of bacteria/PMN	Ruffling[b]	No. of pseudopods/PMN
None	−	0	0.74	0.31
1.1	−	0.15	2.59	6.01
1.2	−	0.32	0.75	1.12
1.3	−	0.27	0.71	2.08
1.1	+	2.60	0.78	0.26
1.2	+	6.37	0.86	1.35
1.3	+	6.73	1.35	1.02
1.5	+	1.03	1.75	2.80

[a] Forty consecutive PMNs were scored by two independent blinded observers.
[b] Scored 0 to 3+.

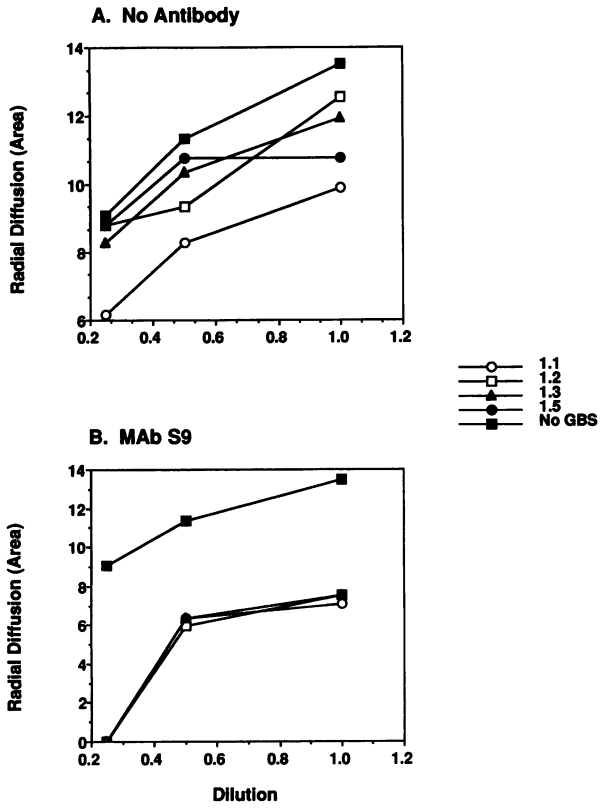

FIG. 6. Complement activation by GBS opacity variants. Activation of complement by GBS was measured as a function of complement consumption from a control serum. GBS opacity variants were first incubated in either the absence (A) or presence (B) of MAb S9. The GBS were subsequently washed and then incubated with the complement-containing serum. Following incubation, the GBS were centrifuged from the serum. Total hemolytic complement was measured in serial dilutions of the serum by radial immunodiffusion in agar plates containing sensitized SRBC. The area of radial diffusion is plotted against serum dilution.

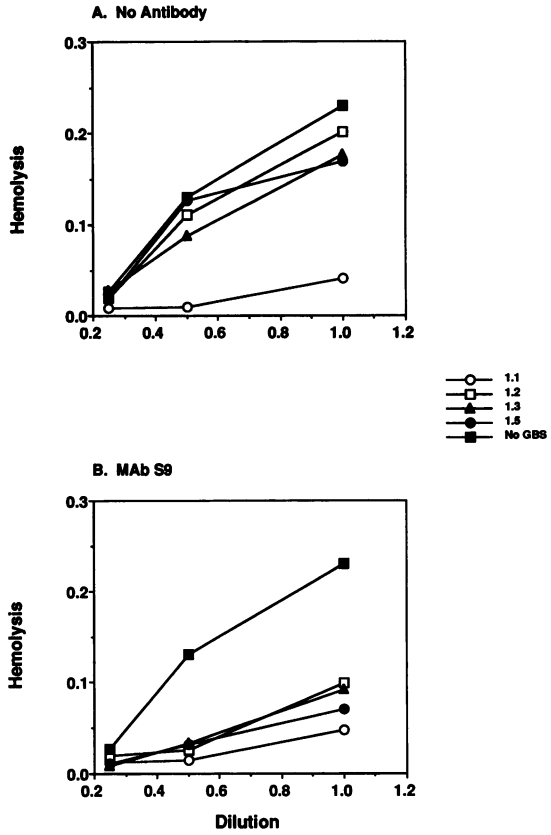

FIG. 7. Complement activation by GBS opacity variants. Complement-containing serum was depleted of complement as described for Fig. 5. To assay for the remaining hemolytic complement in the serum, serial dilutions of the serum were incubated with sensitized SRBC in suspension. Following incubation, the SRBC were sedimented and the A_{514} was determined. The absorbance is plotted against the serum dilution.

removed. However, in the absence of MAb S9, only the variant 1.1 caused any complement consumption, removing approximately 75% of the complement activity. These results were confirmed by using a second, solution-phase assay in which the lysis of sensitized SRBC was measured spectrophotometrically (Fig. 7). Again, in the presence of MAb S9, all strains activated complement, whereas in its absence, only variant 1.1 caused complement consumption. In this assay, the degree of complement consumption by variant 1.1 in the absence of MAb S9 was equal to that produced by the GBS in the presence of the MAb.

In vivo virulence. The in vivo virulence of the GBS opacity variants was tested in neonatal mice. Litters of mice were injected with graded numbers of each of the variants, and their survival was monitored for 1 week. Figure 8 shows the survival curves for the first 3 days, after which time no mortality occurred. The transparent variants, 1.2 and 1.3, produced greater mortality at all concentrations tested than did the opaque variants. Mortality was seen with variant 1.1 at only the highest concentration of GBS, whereas there was no significant mortality with variant 1.5 at any concentration tested. The failure of the opaque variants to cause disease was not due to a lack of invasiveness of the bacteria. The animals were injected intraperitoneally, yet GBS could be cultured from the brain 18 h postinoculation with each of the opacity variants (data not shown). GBS isolated from mice infected with variants 1.1, 1.2, and 1.3 were transparent, while opaque GBS were isolated from variant 1.5-infected mice.

To determine whether interactions between the different opacity variants could enhance virulence, an experiment was performed in which the avirulent variant 1.5 was mixed with low numbers of the more virulent transparent variant 1.2 (Fig. 9). Mice were injected with undiluted variant 1.5, a 10^{-5} dilution of variant 1.2, or a mixture of both. No mortality was seen with variant 1.5 alone, and minimal mortality was seen with variant 1.2 alone, but when the two were mixed, there was a highly significant increase in mortality ($P < 0.005$ by the Wilcoxon rank order test). When GBS were cultured from mice receiving the mixed inoculum, all cultured GBS were transparent. These data indicate that the presence of the avirulent opaque GBS can potentiate the virulence of the transparent form.

DISCUSSION

GBS exhibit high-frequency phase variation between long- and short-chain morphotypes, which can be detected as changes in colony opacity (5). In addition to differences in chain length, the opaque and transparent GBS differ from each other in a number of surface characteristics. Because

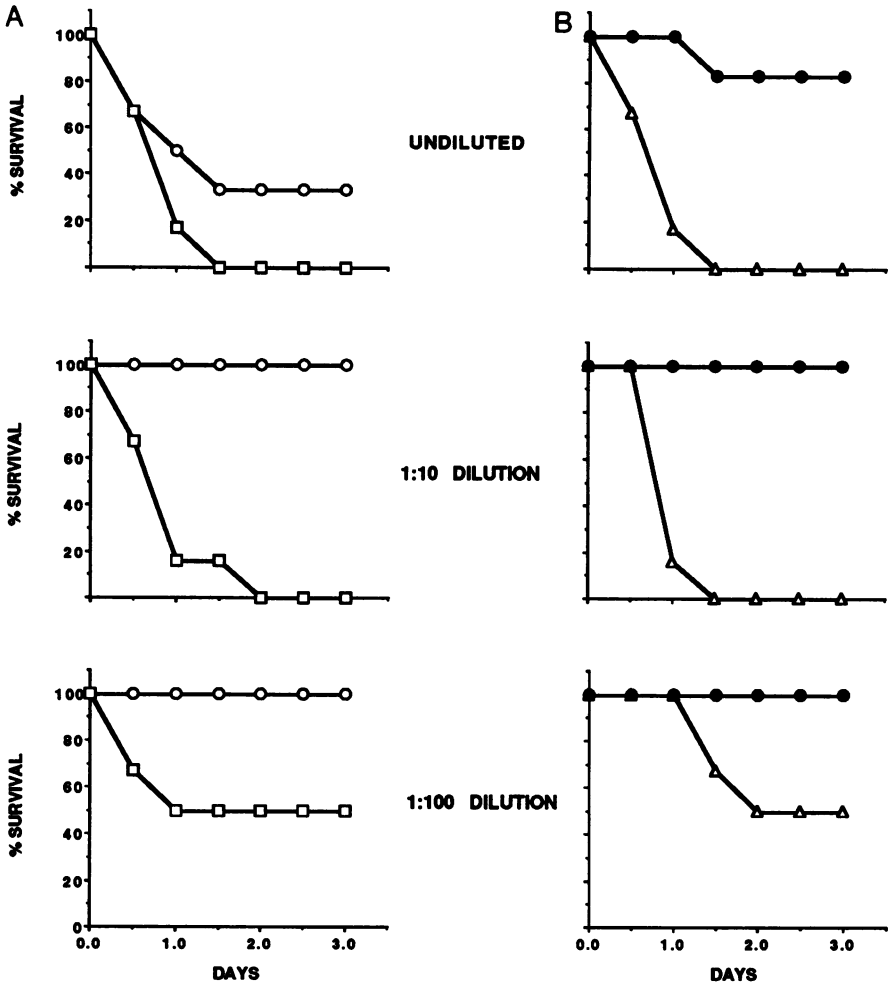

FIG. 8. In vivo virulence of GBS opacity variants. Neonatal mice were infected with serial dilutions (undiluted was 10 μl of a solution with an A_{600} of 0.9) of GBS opacity variants given intraperitoneally. Experimental groups consisted of litters of six mice and a lactating female. Each group was housed in a separate cage. Mice were observed for mortality twice daily for 7 days. The percent survival is plotted versus time. No additional mortality was seen after day 3. (A) Mice infected with variants 1.1 (○) and 1.2 (□); (B) mice infected with variants 1.3 (△) and 1.5 (●).

Todd-Hewitt medium, the standard medium used to grow and study GBS, does not support the growth of opaque forms, the role of opacity phase variation in the pathogenesis of disease caused by GBS has not been studied. In this study, we have evaluated the interactions of GBS opacity variants with components of the host defense system. The data demonstrate that the opaque variants were less immunogenic and less virulent than the transparent forms. The opaque GBS nonspecifically induced PMN activation in the absence of Ab and complement, and opaque variant 1.1 was also able to initiate the complement cascade in the absence of Ab. These differences in host interactions between opaque and transparent GBS may play a role in the ability of GBS to cause disease.

A possible confounding factor in our experiments is that for a given optical density of GBS, the opacity variants yield different numbers of colonies (Table 1); this is primarily a function of chain length (5). In streptococci, a CFU may be as large as a complete chain or a fraction thereof. We chose to use optical density rather than CFU as the basis for comparing the different opacity variants because we felt that this approach most closely equalized the total number of

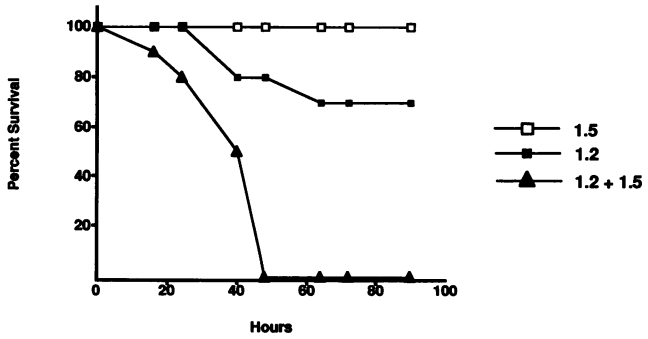

FIG. 9. Effects of mixed infection with different opacity types on virulence of GBS. Neonatal mice were injected with avirulent opaque variant 1.5 (A_{600} of 0.9, undiluted), a low concentration of virulent transparent variant 1.2 (10^{-5} dilution), or a mixture of both. Experimental groups consisted of litters of 10 mice. Mortality was observed for 7 days. The percent survival is plotted against time. The difference between infection with variant 1.2 alone and the mixed infection was highly significant ($P < 0.005$).

individual organisms and the surface area available for immune interactions. In support of this view are ELISA results showing binding of equal amounts of monoclonal anticapsular Ab when bacteria were adjusted to equal optical densities (5). Nevertheless, these assumptions may have biased the in vivo virulence experiments, in which the number of CFU injected may more accurately reflect the ability of bacteria to cause mortality. However, the difference in the number of CFU for a given optical density varies only 10-fold (Table 1), whereas the differences in virulence vary more than 100-fold.

The differences between the opaque and transparent GBS in their interactions with the host defense system may be due to unique surface characteristics of the different variants (5), or they may be due to the different states of aggregation exhibited by the variants. In particular, the in vivo virulence may be affected by chain length, since size can clearly influence factors such as tissue penetration. Moreover, the difference in virulence that we observed was inversely related to chain length. On the other hand, differences in complement activation are not likely to be due to chain length, since the GBS that form the longest (variant 1.5) and shortest (variants 1.2 and 1.3) chains behaved the same, while 1.1, the variant with intermediate chain length, differed. It seems likely that both chain length and cell surface factors account for the differences in the interactions between the GBS variants and host defenses.

The bacterial capsule has been defined as a virulence factor in GBS and has been shown to exert an anticomplement effect (3, 8–10). It is of note that opaque variant 1.1 activates complement in the absence of Ab and has diminished virulence. Variant 1.1 has an altered capsule compared with other type III GBS. Although this variant reacts with anticapsular MAb S9, the total amount of capsular polysaccharide (per gram [dry weight]) is markedly diminished. Moreover, the net surface charge of variant 1.1 is less negative, and the buoyant density in hypotonic buffer is higher than that of the other GBS variants, findings consistent with a diminished capsule (5). These data support the idea that the type III capsular polysaccharide inhibits the activation of complement.

PMN activation can be measured by multiple different criteria. In this study, we have used both morphological criteria and luminol-enhanced chemiluminescence. Morphological criteria of a PMN response (ruffling, pseudopod formation) did not always correlate well with the level of activation as measured by luminol-enhanced chemiluminescence. For example, opaque GBS with Ab and complement produced the highest chemiluminescent response but with little morphological evidence of activation, while variant 1.1 in the absence of Ab and complement induced less chemiluminescence but ruffled the cells greatly (Table 3; Fig. 4 and 5). This finding suggests that the GBS opacity variants activate different functions in PMNs.

Our data indicate that colony opacity can affect virulence in the GBS variants (Fig. 8). Variant 1.1, with a diminished capsule, is less virulent than transparent GBS. Variant 1.5, which has a full capsule, is virtually avirulent. Thus, capsular expression is not the sole factor affecting virulence in these GBS. It seems likely that nonspecific activation of PMNs and (in the case of variant 1.1) complement as well as chain length play a role in the decreased virulence of the opaque GBS. Differences in virulence are only partially related to invasiveness, since opaque GBS, as well as the virulent transparent GBS, can be recovered from distant sites. Virulence can be enhanced by mixing opaque and transparent GBS, although the mechanism for this bacterial synergy is not understood.

The relationship between GBS and the host can vary greatly. The GBS may colonize epithelial surfaces, where they may or may not induce an immune response. They may cause indolent infection or, under appropriate circumstances, a rapidly progressive fatal septicemia. While host factors undoubtedly play a major role in determining the type of interaction, microbial determinants may also influence the outcome. Differences in the interactions between opacity variants and host defenses may account for the ability of GBS to occupy different niches within the host. The data presented here suggest a model to explain the interplay between GBS and their hosts. This model posits that colonization is accomplished by opaque GBS and disease is effected by transparent GBS, and it seeks to explain the transition of GBS from commensal to pathogen as a function of opacity phase variation. The model is easily testable. Clinical isolates of GBS may be examined for opacity status as early after isolation as possible, avoiding passage of the GBS on Todd-Hewitt medium. We are currently collecting such clinical specimens and have found that transparent, opaque, or mixed cultures of GBS can be directly isolated from humans, indicating that differences in opacity of GBS do occur in humans. Future analyses should allow us to determine whether GBS opacity phase variation plays a role in disease pathogenesis.

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REFERENCES

1. **Baker, C. J.** 1977. Summary of the workshop on perinatal infections due to group B streptococcus. J. Infect. Dis. **136:**137–152.
2. **Baker, C. J.** 1986. Group B streptococcal infection in newborns. N. Engl. J. Med. **314:**1702–1704.
3. **Edwards, M. S., D. L. Kasper, H. J. Jennings, C. J. Baker, and A. Nicholson-Weller.** 1982. Capsular sialic acid prevents activation of the alternative complement pathway by type III, group B streptococci. J. Immunol. **128:**1278–1283.
4. **Hemming, V. G., R. T. Hall, P. G. Rhodes, A. O. Shigeoka, and H. R. Hill.** 1976. Assessment of group B streptococcal opsonins in human and rabbit serum by neutrophil chemiluminescence. J. Clin. Invest. **58:**1379–1387.
5. **Pincus, S. H., R. L. Cole, M. R. Wessels, M. D. Corwin, E. Kamanga-Sollo, S. F. Hayes, W. Cieplak, Jr., and J. Swanson.** 1992. Group B streptococcal opacity variants. J. Bacteriol. **174:**3739–3749.
6. **Pincus, S. H., A. O. Shigeoka, A. A. Moe, L. P. Ewing, and H. R. Hill.** 1988. Protective efficacy of IgM monoclonal antibodies in experimental group B streptococcal infection is a function of antibody avidity. J. Immunol. **140:**2779–2785.
7. **Pincus, S. H., and M. R. Wessels.** Unpublished data.
8. **Rubens, C. E., M. R. Wessels, L. M. Heggen, and D. L. Kasper.** 1987. Transposon mutagenesis of type III group B *Streptococcus*: correlation of capsule expression with virulence. Proc. Natl. Acad. Sci. USA **84:**7208–7212.
9. **Shigeoka, A. O., N. S. Rote, J. I. Santos, and H. R. Hill.** 1983. Assessment of the virulence factors of group B streptococci: correlation with sialic acid content. J. Infect. Dis. **147:**857–863.
10. **Wessels, M. R., C. E. Rubens, J. V. Benedi, and D. L. Kasper.** 1989. Definition of a bacterial virulence factor: sialylation of the group B streptococcal capsule. Proc. Natl. Acad. Sci. USA **86:**8983–8987.